Lack of Strict Correlation of Functional Sensitization with the Apparent Affinity of MHC/Peptide Complexes for the TCR[1]

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We describe a comprehensive analysis of the effect of avidity of TCR-MHC/peptide interaction on activation of the alloreactive 2C CTL clone, which recognizes H-2L^d plus an octamer peptide (p2Ca). In this study, monosubstituted variants of p2Ca were used and assessed for binding to purified H-2L^d, binding of H-2L^d/peptide complexes to sTCR, and ability to activate 2C cells to two independent effector functions. Among the >20 variants analyzed, functional activity of most peptides that bound the MHC well correlated with the strength of interaction of MHC/peptide complexes with sTCR. However, with some variants, a clear discordance between the apparent TCR-MHC/peptide affinity and biologic function was observed, demonstrating that the former cannot always be gauged by the latter. In the case of L4 peptide (phenylalanine at position 4 substituted with leucine), peptide/MHC complexes showed no detectable binding to sTCR, indicating a 10-fold or greater decrease in affinity. Nevertheless, this peptide sensitized target cells for lysis at a level equivalent to the parental peptide. A clearer understanding was revealed by studying the extent to which activation by variant peptides was dependent on CD8. Our data indicate that resistance to anti-CD8 mAb blocking correlates with strong binding affinity between sTCR and MHC/peptide complexes. These data suggest that, for the activation of CTL function, the absolute level of intrinsic affinity of TCR for MHC/peptide ligand is not a single critical determinant, but rather, that activation is governed by the compound influence of several factors, which ensures a minimum threshold of intracellular triggering is reached to elicit the response. *The Journal of Immunology*, 1995, 155: 662–673.

The activation of CD8$^-$ CTL typically involves the recognition by their Ag-specific TCR-$\alpha\beta$ of peptidic fragments presented in the context of MHC class I molecules (1). The recent advances made in the resolution of the structure of MHC class I proteins by x-ray crystallography (2–4), coupled with the ability to directly elute and sequence MHC-bound peptides (5–7), have provided an increasingly detailed view of the characteristics of MHC/peptide interaction at the molecular level. MHC class I-associated peptides are generally 8 to 10 amino acids in length and they bind in an extended conformation to a groove formed by the α1 and α2 domains of class I molecules. Since TCR-MHC/peptide interaction is central both to the development of the T cell repertoire and to Ag-specific activation of mature T lymphocytes, it has been of considerable interest to devise strategies to determine the characteristics of the interaction between TCR and the MHC/peptide complex. Several laboratories, including our own, have reported quantitative estimates of the strength of TCR-MHC/peptide interactions (8–12). Distinct from other reported studies, our strategy is based on the use of an engineered purified form of a well-characterized, class I-restricted TCR, derived from the alloreactive 2C CTL clone, which recognizes an octamer self-peptide, known as p2Ca (LSPFPFDL), in association with H-2L^d (13, 14). Using a surface plasmon resonance detection system, we have recently demonstrated that this interaction is of moderate affinity, with a

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K_d of $\sim 10^{-7}$ M, and is characterized by moderate association and rapid dissociation kinetics (12).

It is generally accepted that the CD8 co-receptor plays an important role in CTL maturation and function (15). Mature T cells express CD8 in the form of a disulfide-linked $\alpha\beta$ heterodimer that binds to a nonpolymorphic region in the $\alpha 3$ domain of MHC class I molecules (16, 17), thereby increasing the overall avidity of TCR-MHC peptide interaction. In the case of the 2C TCR, however, two recent observations suggest that the requirement for CD8 is not absolute. In 2C TCR transgenic mice, thymocytes bearing the 2C TCR have been shown to be positively selected on H-2K^b and negatively selected on H-2L^d class I molecules (18, 19). Using 2C TCR transgenic mice crossed with CD8α-deficient mice, it was demonstrated that H-2L^d-mediated negative selection of 2C cells does not require CD8 expression (20). In the same study, negative selection of two other class I-restricted TCR was shown to be CD8-dependent (20). Furthermore, in another study, both CD8$^-$ and CD8$^-$ 2C TCR$^-$ transgenic T cells were shown to respond to H-2L^d-expressing targets equally well (21). These results have been interpreted to mean that the 2C TCR has a relatively high affinity for its H-2L^d peptide ligand, an interpretation that has now been confirmed by direct measurement of TCR affinity (11, 12). In contrast with those studies that utilized 2C TCR transgenic mice, earlier experiments demonstrated that, for the mature 2C CTL clone, recognition of H-2L^d-bearing target cells was inhibited by anti-CD8 mAb (22).

In an effort to fully understand the characteristics of 2C TCR-MHC peptide recognition at the molecular level, and taking advantage of the availability of 2C sTCR, we undertook a detailed analysis of this interaction using synthetic peptides containing single amino acid substitutions at each position of the p2Ca peptide moiety. The panel of peptides was analyzed for binding to soluble H-2L^d (sH-2L^d), the binding of MHC/peptide complexes to purified 2C TCR, the ability to sensitize for cytolysis as well as IL-3 production by the 2C CTL clone, and the dependence of peptide-specific lysis on CD8. Surprisingly, our results indicate a discordance between the ability of some variant peptides, which form complexes, to bind the TCR and to stimulate the T cell. Furthermore, the data provide a direct demonstration that, for a given interaction, CD8-dependency is a function of two parameters: the intrinsic affinity of TCR to its MHC/peptide ligand and ligand density. In addition, the paradoxical behavior of several peptide variants suggests that molecular events besides TCR binding of the MHC/peptide complex are required for T cell activation.

Materials and Methods

Cell lines and mAbs

The alloreactive 2C CTL clone, specific for H-2L^d, was derived from a BALB.B (H-2^b) mouse injected with H-2^{bd} cells (23). 2C cells recognize an octamer self-peptide, designated p2Ca, derived from the housekeeping enzyme 2-oxoglutarate dehydrogenase, in the context of H-2L^d (13, 24). 2C cells were grown in modified Dulbecco's MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10 mM HEPES, 4 mM L-glutamine, 1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM 2-ME, and 5% (v/v) heat-inactivated FCS. They were maintained by weekly stimulation with irradiated DBA.2 (The Jackson Laboratory, Bar Harbor, ME) spleen cells in the presence of 25 to 50 U/ml of IL-2 culture supernatant obtained from the constitutively-secreting IL-2-transfected TCX6310-IL2 myeloma line (25). The C1R L$^-$ line (26) is a derivative of the human MHC class I-negative B lymphoblastoid cell line C1R transfected with H-2L^d genomic clone, and was the kind gift of P. Cresswell (Section of Immunobiology, Yale University Medical School, New Haven, CT). It was maintained in Iscove's modified Dulbecco's MEM supplemented with 5% FCS. The IL-3 GM-CSF$^-$-dependent cell line FDC.P1 (27, 28), kindly provided by J. N. Ihle (Yale University), was maintained in Click's EHAA medium with 10% FCS and IL-3-containing culture supernatant. The mAbs used were 30–5–7s (specific for the $\alpha 2$ domain of H-2L^d) (29), 53–6.7 (30), and 2.43 (31), which are specific for murine CD8α and CD8α.2, respectively. Hybridoma cell lines secreting the above mAbs were obtained from American Type Culture Collection, Rockville, MD.

Peptides

All peptides used in this study (see Table I) were synthesized and purified as previously described (7) and were provided by John Coligan (Laboratory of Molecular Structure, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Peptides are referred to by the standard single letter code.

Soluble H-2L^d protein

sH-2L^d was produced in transfected L cell lines and immunoaffinity purified as described previously (32, 33). sH-2L^d consists of the $\alpha 1$ and $\alpha 2$ domains of H-2L^d covalently linked to the $\alpha 3$ domain and carboxyl terminus of the class I-like molecule Q10^b, noncovalently assembled with the light chain, β_2-microglobulin.

Epitope induction assay

The mAb 30–5–7s recognizes a peptide-dependent conformational epitope of the $\alpha 2$ domain of maturely folded H-2L^d (34). Binding of peptides to sH-2L^d was detected by real time surface plasmon resonance (SPR) using BIAcore (Pharmacia Biosensor, Piscataway, NJ) as previously described (7). Briefly, purified sH-2L^d was incubated with different concentrations of p2Ca or variant peptides for 1 to 2 h at room temperature to achieve equilibrium. The complexes were then analyzed for direct binding to immobilized 30–5–7s mAb. The increase in resonance units (RU) is directly proportional to the mass of sH-2L^d peptide complexes associated with the mAb. To derive quantitative estimates for the strength of binding, these data were curve fitted to the four parameter sigmoidal expression,

$$y = \{(a - d)/(1 + |x/c|^b)\} - d,$$

where a is the minimal RU; b is the slope factor; c is the value of x with half-maximal binding; and d is the maximal RU, with the use of Kaleidagraph (Synergy Software, Reading, PA).

TCR binding assay

2C sTCR was produced in transfected thymoma cell line BW5147 as previously described (12, 14). Purified 2C TCR was coupled to the Pharmacia BIAcore dextran-modified surface by amine chemistry as described (12). Purified sH-2L^d molecules (1 μM; unless otherwise indicated), exposed to an excess of the indicated peptides (250 μM), were injected at a flow rate of 5 μl/min for 8 min each.

The surface was regenerated with HBST (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.05% Tween 20). p2Ca and pMCMV (YPH

Abbreviations used in this paper: GM-CSF, granulocyte-macrophage colony-stimulating factor; SPR, surface plasmon resonance; RU, resonance units; BD_{50}, binding dose required for 50% of maximal binding; AD_{50}, 50% activation dose.

FMPTNL) peptides were used as positive and negative controls, respectively. pMCMV binds strongly to sH-2L^d but the complex is not bound by the 2C TCR (12). All peptides were diluted in double-distilled H_2O except for A2, D1, and Y1, which were solubilized in the presence of acetic acid. TCR binding to the latter peptides was conducted in separate experiments in which they, together with the control p2Ca and pMCMV peptides, were diluted similarly in acetic acid. All measurements were expressed in RU and performed at 25°C. The percentage binding for each sH-2L^d peptide complex to immobilized 2C TCR was expressed as

$$\{RU(sH\text{-}2L^d\text{-peptide}) - RU(sH\text{-}2L^d\text{-pMCMV})\}$$

$$\{RU(sH\text{-}2L^d\text{-p2Ca}) - RU(sH\text{-}2L^d\text{-pMCMV})\} \times 100.$$

In some cases, binding curves were obtained with a large excess of the indicated peptides (500 μM), and a titration of sH-2L^d. Maximal binding was corrected for the refractive index change due to the sH-2L^d protein itself, and plotted as a function of sH-2L^d concentration. These data were fit to the four-parameter sigmoidal expression described above.

Cytotoxicity assay

Target lysis by 2C cells was determined using the JAM test, an assay that measures CTL activity as a function of the degree of DNA degradation in the target cells rather than membrane integrity (35). Briefly, 1×10^6 target cells were labeled with 5 μCi of [^{3}H]TdR for 3 to 4 h, washed, and plated at 10^4 cells well (in 50-μl volume) in round-bottom, 96-well plates containing 50 μl of PBS or test peptides diluted in PBS.

After 30 min incubation at 37°C, 2C CTL (at 3×10^5 ml) were added in 100 μl of complete Dulbecco's MEM giving a final E:T ratio of 3:1. The assay plates were maintained in a 37°C incubator with 5% CO_2 for 2 to 4 h, after which they were harvested and counted on a beta counter. Percent specific lysis was calculated as:

$$100 \times \{\text{spontaneous DNA retention (cpm)} - \text{experimental DNA retention (cpm)}\}.$$
$$\{\text{spontaneous DNA retention (cpm)}\}:$$

where spontaneous and experimental retentions reflect the [^{3}H]TdR-labeled DNA in targets incubated without or with CTL, respectively. Specific lysis in the absence of added peptides, typically <5%, was routinely subtracted.In experiments to test the effect of adding anti-CD8 mAb, target cells were preincubated with test peptides, as above, and both the anti-CD8 mAb (in 50 μl) and 2C CTL (50 μl of 6×10^5/ml cell suspension) were then added simultaneously to the assay wells. The assay plates were then incubated and processed as described above. Percent inhibition of lysis was determined as:

$$100 \times \{(A - B)/(A)\}$$

where A is the percent specific lysis in the absence of anti-CD8 mAb, and B is the percent lysis in its presence. Conversely, this data can be expressed as the percent of maximum response calculated as: 100 − percent inhibition of lysis.

IL-3 assay

C1R L^d stimulator cells were irradiated (20,000 rad), washed three times, and 1×10^5 cells in 50 μl were added to wells of a 96-well, round-bottom plate containing 50 μl of titrated concentrations of the test peptide. Following a 30-min incubation at 37°C, 1×10^5 2C cells (in 100 μl) were added. After a 24-h incubation the plate was centrifuged briefly, and 100 μl of supernatant was removed and subjected to two rounds of freeze-thaw. The indicator cell line FDC.P1 was washed thoroughly in PBS, resuspended in Click's EHAA + 10% FCS medium at a concentration of 2×10^5 cells/ml, and added in 100-μl volume to plates containing the test supernatants. Following a 48-h incubation at 37°C, the wells were pulsed with 1 μCi of [^{3}H]TdR for a further 16 to 20 h, then harvested and counted. It is important to note that the FDC.P1 cell line proliferates to both IL-3 and GM-CSF (28), and alloreactive CD8$^+$ CTL have been shown to secrete both lymphokines (36). Since it is not central to this study, we did not attempt to distinguish between these two lymphokines; however, for the sake of clarity, this assay will be referred to as IL-3 secretion assay.

Table I. *List of peptides used in this study*

Peptide designation	Amino Acid sequence							
p2Ca	L	S	P	F	P	F	D	L
A1	A	–	–	–	–	–	–	–
A2	–	A	–	–	–	–	–	–
A3	–	–	A	–	–	–	–	–
A4	–	–	–	A	–	–	–	–
A5	–	–	–	–	A	–	–	–
A6	–	–	–	–	–	A	–	–
A7	–	–	–	–	–	–	A	–
A8	–	–	–	–	–	–	–	A
V1	V	–	–	–	–	–	–	–
Y1	Y	–	–	–	–	–	–	–
I1	I	–	–	–	–	–	–	–
K1	K	–	–	–	–	–	–	–
D1	D	–	–	–	–	–	–	–
Y4	–	–	–	Y	–	–	–	–
L4	–	–	–	L	–	–	–	–
C4	–	–	–	C	–	–	–	–
E7	–	–	–	–	–	–	E	–
K7	–	–	–	–	–	–	K	–
T7	–	–	–	–	–	–	T	–
N7	–	–	–	–	–	–	N	–

[a] Name of peptide is denoted by the name of the amino acid substitution in single-letter code followed by the position of the substituted residue.
[b] Dashes indicate identity with the p2Ca peptide.

Results

To study the contribution of each peptide residue to H-2L^d binding and CTL recognition, we tested alanine (A = single letter amino acid code)-monosubstituted mutant peptides of p2Ca (for a list of peptides, refer to Table I) for their capacity to both bind sH-2L^d and sensitize H-2L^d-expressing target cells for lysis by 2C cells. Binding of the various peptides to sH-2L^d was determined by their capacity to induce a conformational epitope in H-2L^d detectable by the conformation-specific mAb 30–5–7s, using real time SPR. As shown in Figure 1, with the exception of A6 and A8, all other variant peptides bound H-2L^d strongly. To obtain quantitative estimates for the strength of binding, the data were curve fitted, as described in *Materials and Methods*, and the dose required for half-maximal binding (BD_{50} = binding dose required for 50% of maximal binding) was derived (shown in parentheses in Fig. 1). (Similar results with the same rank order of BD_{50} were obtained in a second independent assay in which these peptides were used to inhibit the direct binding of sH-2L^d to immobilized peptide (32) (data not shown).) Based on this analysis, it is clear that peptides A5 and A4 exhibited the strongest binding, with BD_{50} values ~4 to 16 times lower than p2Ca. Peptides A1 and A3 bound sH-2L^d almost identical to p2Ca, and A2 and A7 peptides were slightly less effective than p2Ca. The weakest binding to sH-2L^d was exhibited by A6 and A8 peptides, requiring ~6 to 20-fold higher concentration of peptide for half-maximal binding as compared with p2Ca.

We then analyzed the ability of these mutant peptides to be recognized by 2C cells in a direct lysis assay (Fig. 2).

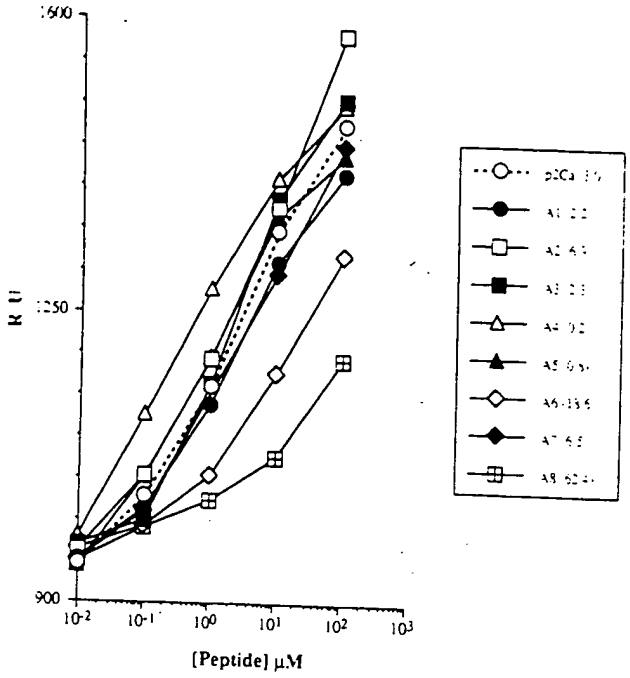

FIGURE 1. Binding of p2Ca or A-substituted variant peptides to sH-2L^d as assessed by an epitope-induction assay sH-2L^d was exposed to graded concentrations of the indicated peptides and then passed over a biosensor surface coupled to the mAb 30-5-7s as described in *Materials and Methods*. Binding values are reported as RU, which were fitted to the four parameter equation described in *Materials and Methods*. For each peptide, the concentration (in μM) giving half-maximal binding is indicated in parentheses.

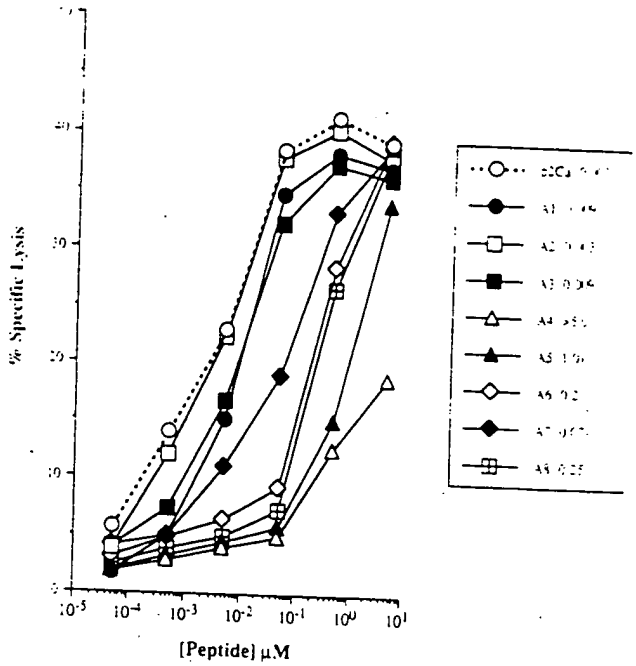

FIGURE 2. Lysis of C1R/L^d targets by 2C CTL in the presence of various concentrations of p2Ca or A-substituted variant peptides at E:T ratio of 3:1. All cultures were set up in triplicate. The peptide doses (in micromolar quantities) yielding half-maximal lysis (AD_{50} values) are shown in parentheses. Background lysis, in the absence of added peptides, was 4.5%.

As targets, we used a human lymphoblast cell line transfected with mouse H-2L^d (C1R/L^d). We have previously determined that at low E:T ratios, this line is not lysed by 2C cells in the absence of added peptide (data not shown), thus providing a convenient way of assessing peptide-specific lysis. The results of the CTL-sensitization assay demonstrate several points. First, at an E:T ratio of 3:1, the p2Ca peptide sensitizes targets for 50% lysis (AD_{50} = activation dose) at a peptide concentration of ~3 nM. Since the affinity of the peptide for sH-2L^d, as estimated both by epitope induction and competitive assays, is ~0.1 to 1 × 10^{-6} M (data not shown), this would suggest that as little as 0.03 to 0.3% of the MHC class I molecules need to be occupied for sensitization. Second, peptide A2 sensitized indistinguishably from p2Ca peptide, and peptides A1 and A3 were slightly less effective, all with 50% lysis falling in the 3- to 10-nM range. Third, as would be expected, peptides A6 and A8, which are weak H-2L^d-binders, were also recognized less efficiently by 2C cells with AD_{50} in the 200- to 300-nM range. Finally, although A4 and A5 peptides bound sH-2L^d strongly, they were the weakest in sensitizing the targets for lysis by 2C cells, with AD_{50} of >5000 nM and 1000 nM, respectively. Similarly, a significantly weaker sensitization capacity was observed with the A7 mutant peptide (AD_{50} ~70 nM).

Recognizing that CTL lysis represents a highly sensitive assay for examining the functional activities of peptides, we analyzed the properties of the variant peptides using a second independent biologic assay, namely, the ability to induce the secretion of IL-3 by 2C cells. It was found that the pattern of responses observed in the lysis assay was essentially similar to that in the IL-3 secretion assay (data not shown; refer to Table II for summary). Taken together, the data demonstrate that A substitution at position 1 or 2 has little or no effect on the recognition as assessed by either of the two functional assays, while A substitution at each of the other positions significantly impairs recognition. In the case of A6 and A8 peptides, the decreased activity is probably due to their significantly weaker binding to sH-2L^d; thus, for p2Ca, it appears that positions 6 and 8 are important for peptide binding to H-2L^d, and that positions 3, 4, 5, and 7 are critical for T cell recognition.

We next sought to correlate the functional data with the direct binding of sTCR to sH-2L^d/peptide complexes. sH-2L^d was incubated with the various peptides and then passed over immobilized 2C sTCR on the biosensor surface. Surprisingly, of the eight alanine-mutant peptides, only A1 and A2 formed complexes with sH-2L^d that were detectably bound by the TCR (Fig. 3). Remarkably, peptide A3, which binds sH-2L^d strongly and sensitizes well for cytolysis, failed to form complexes capable of detectably binding the TCR in this assay. The degree of binding

Table II. A summary of peptide properties[a]

Peptide	H-2L[d] Binding	Lysis Assay	IL-3 Assay[b]	Anti-CD8 mAb Inhibition[c]	sTCR Binding[d]
Group I					
p2Ca	3+	3+	3+	−/−	2+
A1	3+	3+	3+	−/−	1+
V1	3+	3+	3+	−/−	2+
I1	3+	3+	3+	−/−	2+
Y1	3+	3+	3+	−/−	2+
K1	3+	3+	3+	− −	− −
D1	3+	2+	2+	−/−	1+
Y4	3+	3+	3+	− −	2+
Group II					
A2	2+	3+	3+	1+	1+
L4	3+	3+	3+	1+	−
E7	2+	3+	2+	1+	− −
N7	3+	2+	1+	1+	−
Group III					
A3	3+	3+	1+	2+	−
A4	3+	− −	−/−	NA	−
C4	3+	− −	−/−	NA	−
A5	3+	1+	−/−	2+	−
A6	2+	2+	−/−	2+	−
A7	2+	2+	1+	2+	−
K7	1+	−/−	−/−	NA	−
T7	2+	−/−	−/−	NA	−
A8	1+	2+	1+	2+	−

[a] Peptides are grouped based on the susceptibility of peptide-induced CTL lysis response to inhibition by anti-CD8 mAb.

[b] BD_{50}: 3+ = ≤1 to 5 μM; 2+ = >5 to 20 μM; 1+ = >20 μM.

[c] AD_{50}: 3+ = 1 to 20 nM; 2+ = >20 to 400 nM; 1+ = >400 to 4000 nM; − − = >4000 nM.

[d] Percent of p2Ca response: 3+ = >80%; 2+ = >40 to 80%; 1+ = >10 to 40%; − − = ≤10%.

[e] Percent blocking at a peptide dose of 5 μM: 2+ = >70%; 1+ = >20 to 70%; −/− = ≤20%; NA = not applicable due to nearly background levels of lysis in absence of mAb.

[f] Binding to sTCR as a percentage of degree of binding observed with p2Ca: 2+ = >50%; 1+ = >20 to 50%; −/− = 1 to 20%; − − = <1%.

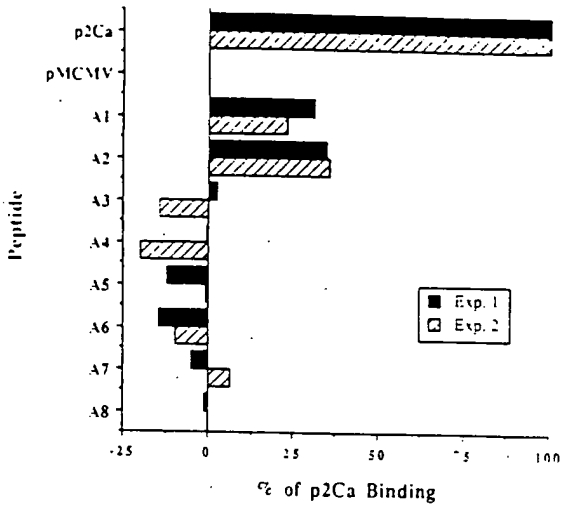

FIGURE 3. Binding of sH-2L[d]/peptide complexes to immobilized 2C TCR. sH-2L[d] equilibrated with a large molar excess of p2Ca, pMCMV, or the indicated variant peptides was passed over a biosensor surface coupled to purified 2C TCR, as described in *Materials and Methods*. Plateau levels of binding of different peptide/sH-2L[d] complexes were expressed as percent of binding of p2Ca/sH-2L[d] to the immobilized 2C TCR.

observed with both the A1 and A2 peptide/sH-2L[d] complexes (27% and 35%, respectively) was significantly weaker when compared with the level of binding observed with complexes formed with p2Ca. These results highlight the extreme sensitivity of the cytolytic response of CD8[+] T cells in being able to lyse targets with an apparently comparable efficiency in the face of varying intrinsic TCR affinities. Additionally, the finding that the A3 peptide is a strong agonist in the lysis assay although the complex is not detectably bound by the TCR under these conditions demonstrates an apparent lack of direct correlation between TCR affinity and the induction of cytolytic response.

To study the extent of this discordance, we examined additional variant peptides with single amino acid substitutions at position 1 and at two key TCR-contact sites, positions 4 and 7 (see Table I for a list of the peptides). These variant peptides were tested for their ability to bind sH-2L[d], sensitize target cells for lysis, and induce secretion of IL-3 by 2C cells. The results obtained with position 1-substituted peptides are shown in Figure 4. In the epitope-induction assay, V1, Y1, and I1 peptides bound sH-2L[d] at levels better than, or equal to, p2Ca (Fig. 4*A*). However, two peptides (D1 and K1), while still showing strong binding, were two to three times weaker in comparison with p2Ca. A similar trend of activity was observed when these peptides were tested in CTL lysis assay. Thus, when the wild-type residue, L, at position 1 of p2Ca peptide was substituted with other hydrophobic residues (V or I), or with the aromatic Y, the variant peptide sensitized for lysis at equivalent or even lower concentrations (Fig. 4*B*). However, significant differences in the dose required for half-maximal sensitization were noted for the two variant peptides, K1 and D1, which required approximately 10-fold and 100-fold more peptide, respectively, for equivalent sensitization (Fig. 4*B*). Qualitatively similar results were obtained when the variant peptides were used to elicit IL-3 production from 2C cells (Table II). Thus, the introduction of a charged basic (K1) or acidic (D1) residue at position 1 of p2Ca appears to decrease the efficiency of binding to sH-2L[d] and, consequently, results in weaker activities in functional assays. These data suggest that the primary influence of the N-terminal residue of p2Ca is on stabilizing the binding to H-2L[d] molecules; however, the apparent discrepancy in the behavior of peptide D1 should be noted, namely that although its binding to H-2L[d] is decreased by 3- to 10-fold compared with p2Ca, it sensitizes for cytolysis 100-fold less efficiently.

Analysis of position 4 variant peptides gave a completely different picture (Fig. 5). In addition to A4, three more variant peptides (C4, L4, and Y4) were tested. All of these variant peptides bound sH-2L[d] stronger than p2Ca (Fig. 5*A*), but they exhibited clear differences in function. The activities of Y4 and L4 peptides were very comparable to p2Ca in both lysis (Fig. 5*B*) and IL-3 (Table II)

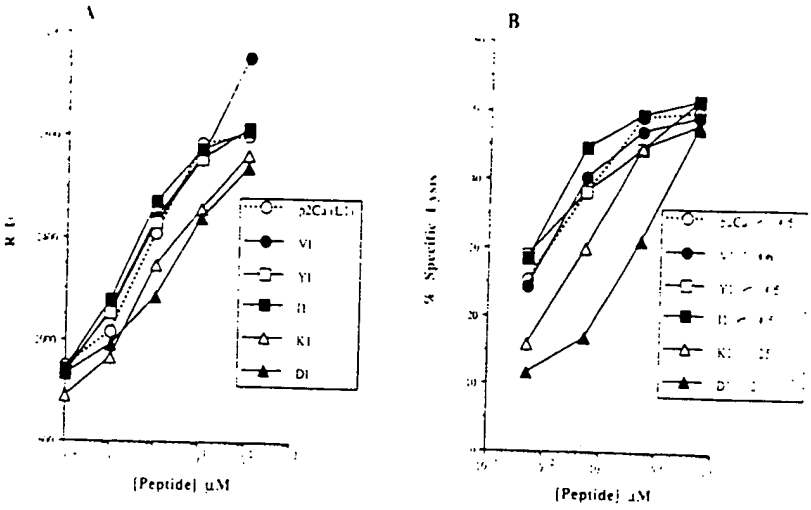

FIGURE 4. Binding and recognition of position 1 variant peptides of p2Ca. *A*, peptide binding to sH-2L^d was assessed by an epitope induction assay as described in the legend to Figure 1 and *Materials and Methods*. The amino acid residue at position 1 of p2Ca peptide is shown in parentheses. *B*, target sensitization for 2C cytolytic activity; E:T ratio = 3:1. Background lysis, in the absence of added peptides, was 1.2%. The AD_{50} values (in micromolar quantities) are shown in parentheses.

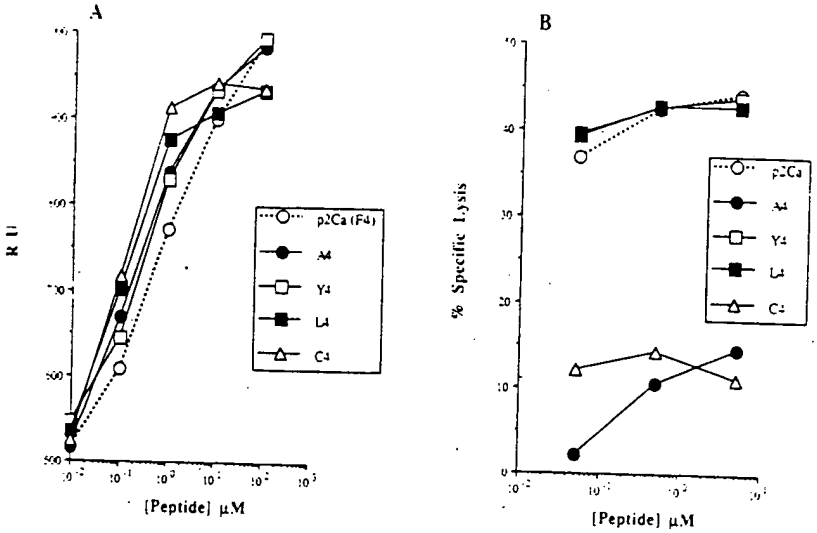

FIGURE 5. Binding and recognition of position 4 variant peptides of p2Ca. *A*, peptide binding to sH-2L^d was assessed by an epitope induction assay as described in the legend to Figure 1 and *Materials and Methods*. The amino acid residue at position 4 of p2Ca peptide is shown in parentheses. *B*, target sensitization for 2C cytolytic activity; E:T ratio = 3:1. Background lysis was 3.8%.

assays. Like the A4 peptide, however, C4 was almost completely inactive in both assays; thus, substituting the wild-type residue, F, with amino acids representing conservative substitutions (Y) or carrying an equally bulky nonpolar side chain (L) are recognized very efficiently by 2C TCR. On the other hand, substituting with either A or C led to a total ablation of function. These results confirm that position 4 is a dominant TCR-contact site.

The results obtained with position 7 mutant peptides indicate that although the major role of this residue is in TCR recognition it is clear that it also has a secondary effect on the efficiency of binding to H-2L^d (Fig. 6). Of the five position 7 variant peptides tested (N7, A7, T7, E7, and K7), only N7 bound sH-2L^d at a level comparable to p2Ca (Fig. 6*A*). The remaining variants bound sH-2L^d with BD_{50} doses ~2-, 6-, 10-, and 33-fold higher than p2Ca, respectively. A different pattern of reactivities was observed when these peptides were tested in the lysis assay. As shown in Figure 6*B*, relative to p2Ca peptide, the AD_{50} doses for the above variants were 20-, 23-, >1500-, 4-, and >1500-fold higher, respectively. As previously observed, the effect of the various substitutions on functional activity, although qualitatively similar, was more drastic in the IL-3 secretion assay (Table II). These results indicate that the pattern of functional activities of the various peptides did not always correlate with their H-2L^d-binding efficiencies. Thus, despite significantly decreased binding, the E7 peptide showed the strongest activity. Similarly, N7 bound sH-2L^d better than p2Ca, however, it was a much weaker agonist in biologic assays. In contrast, the two functionally weakest peptides, K7 and T7, were also the least efficient H-2L^d binders, suggesting that, at least for these two variants, decreased binding to sH-2L^d may be partially responsible for their reduced biologic activities. Therefore, despite having their major influence on TCR recognition, some position 7 substitutions can also affect peptide binding to sH-2L^d.

Having analyzed the functional properties of position 1, 4, and 7 variant peptides, we next examined the binding of their MHC/peptide complexes to sTCR as a means of ascertaining the relative binding affinities. As shown in Figure 7 and Table II, all of position 1 variant peptides formed

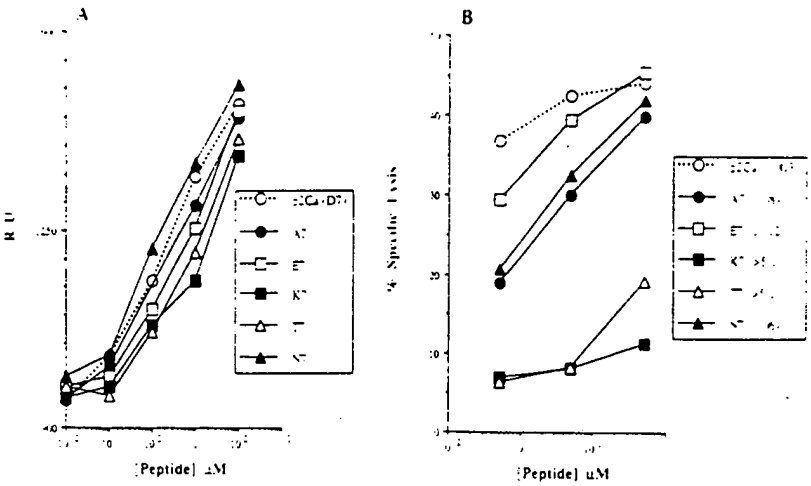

FIGURE 6. Binding and recognition of position 7 variant peptides of p2Ca. *A*, Peptide binding to sH-2L^d was assessed by an epitope induction assay as described in the legend to Figure 1 and *Materials and Methods*. The amino acid residue at position 7 of p2Ca peptide is shown in parentheses. *B*, Target sensitization for 2C cytolytic activity: E:T ratio = 3:1. Background lysis was 5.6%. The AD_{50} values in micromolar quantities are shown in parentheses. Note that for p2Ca, the value given is from Figure 2, and for E7 peptide, the value was derived from an independent experiment in which lower peptide concentrations were used (data not shown).

complexes that were bound by the 2C sTCR, but to varying degrees. Representative binding profiles for p2Ca, V1, I1, A1, and K1 as compared with the negative control pMCMV peptide are shown in Figure 7*A*, and a summary of these data drawn from titrations are in Figure 7*B*. Peptides V1, I1, and Y1 exhibited the strongest binding (52 to 110% of p2Ca binding), A1 and D1 were intermediate (23 to 32% of p2Ca binding), and K1 was the weakest (12 to 31% of p2Ca binding). In Figure 7, *A* and *B*, the p2Ca and variant V1 peptides have almost superimposable sensograms. Of the position 4 variant peptides, only Y4 was detectably bound by the TCR and the level of binding approached that seen with p2Ca (Fig. 7, *C* and *D*). As for position 7 analogue peptides, only E7 bound detectably to the TCR, but the degree of binding was very weak (12 to 14% of p2Ca binding; Fig. 7*E*).

In an effort to evaluate more carefully the relative affinity of the complexes formed with p2Ca, Y4, A1, K7, and L4, kinetic binding curves of the complexes for the immobilized 2C TCR were gathered for each of these over a range of sH-2L^d concentrations (Fig. 7*D*). The equilibrium levels of binding were corrected for background refractive index changes and plotted as a function of concentration (Fig. 7*D*). Half-maximal values obtained by curve fitting were 2.7×10^{-6} M ($\pm$ 3.8×10^{-7}), 2.9×10^{-6} M ($\pm$ 2.3×10^{-7}), and 7.4×10^{-6} M ($\pm$ 1.5×10^{-6}) for p2Ca, Y4, and A1 complexes, respectively. Values obtained in these experiments reflect a lower affinity than measured previously (12), and may be indicative of variability of different preparations of both sTCR and sH-2L^d; however, relative values have always been consistent and the relative affinities of Y4/sH-2L^d and A1/sH-2L^d, as compared with p2Ca/sH-2L^d, complexes for the 2C TCR are 0.93 and 0.36, respectively. These relative values correlate well with the relative degrees of sensitization for lysis induced by the two peptides.

Repeated attempts to demonstrate binding above background of K7 and L4 complexes to 2C TCR have been unsuccessful. Strikingly, although L4 sensitized for cytolysis at a level equivalent to that of Y4, no binding of the L4/sH-2L^d complexes has been detected even at high concentrations. From the data shown in Figure 7*D*, as well as from the analysis of kinetic curves obtained over a wide range of concentrations, we can only establish a limit for the relative affinity of L4/sH-2L^d complexes for 2C TCR to be less than 0.09 that of the affinity of p2Ca complex. Moreover, K7/sH-2L^d complexes did not reproducibly show binding, above background controls, to the 2C TCR. In some cases (Fig. 7*E*), an increase in RU was observed, but the square form of both the association and dissociation phases suggested that this was due to bulk refractive index changes rather than true binding. A summary of the properties of the peptide variants complexed to sH-2L^d in their binding to 2C TCR is shown in Figure 7*E*.

The above data highlight two points. First, peptides whose complexes were strongest bound by the TCR (V1, I1, Y1, and Y4) have wild-type levels of functional activities in both the lysis and IL-3 assays. However, complexes of other peptides that also induced strong or intermediate biologic responses were either poorly bound (K1 and E7) or not bound at all (L4 and A3). Second, complexes of peptides with weak functional activities in one or both assays were not bound by sTCR (e.g. A4, C4, A7, and N7). These data demonstrate that the properties of peptides observed in functional assays do not always correlate with the apparent affinity of interaction of their respective MHC complexes with the sTCR. In some cases, the apparently strong activities observed in functional assays, in the face of low intrinsic TCR affinities, may be due to the involvement of the CD8 co-receptor and/or other accessory molecules.

The results of the functional assays failed to offer a mechanistic explanation for the different classes of substituted peptides, i.e., those that activate in both functional assays and whose complexes bind the TCR vs those that also activate well but do not bind the TCR.

Since susceptibility to anti-CD8 mAb has been used to discriminate between high and low avidity TCR-MHC/

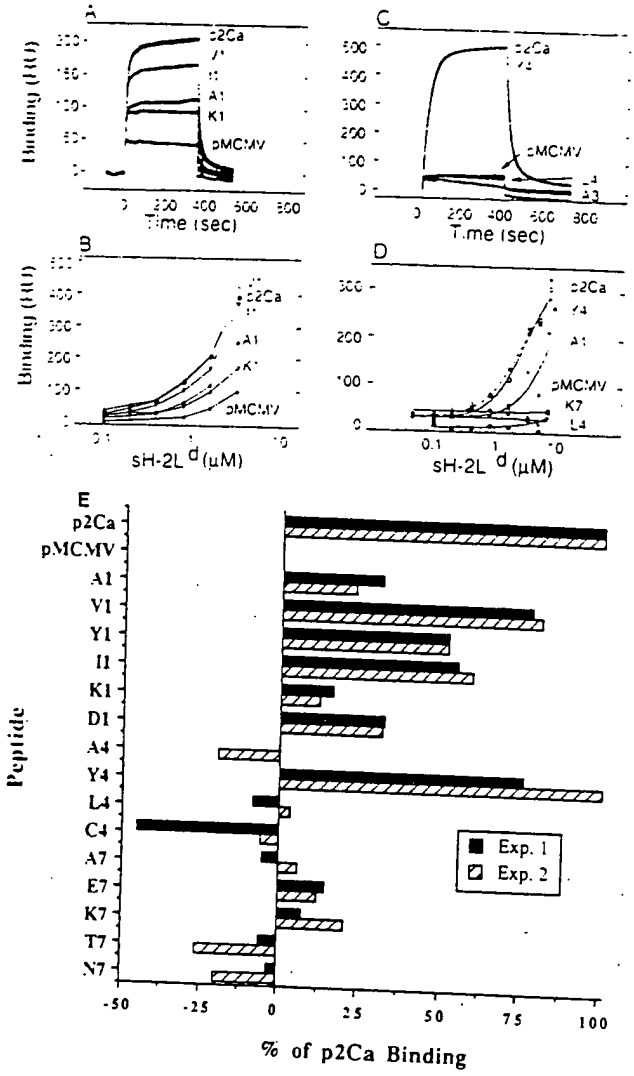

FIGURE 7. Binding of sH-2L^d/peptide complexes to immobilized 2C TCR. *A*, sH-2L^d was equilibrated with the indicated peptides at large molar excess (250 μM), and the complexes were passed over a biosensor surface coupled to purified 2C TCR, as described in *Materials and Methods*. Representative sensograms at a sH-2L^d concentration of 1.6 μM are shown. Injection of the solution phase ligand was initiated at 0 s at a flow rate of 5 μl/min. Washout to evaluate the dissociation phase began at 360 s and was carried out at a flow rate of 100 μl/min. *B*, maximal observed binding at the indicated concentrations of sH-2L^d is plotted for each of the peptides. sH-2L^d concentration is not corrected for the available peptide-binding sites (measured to be 35% of the total sH-2L^d). *C*, sH-2L^d (at 1.0 μM) complexed with the indicated peptides (at 250 μM) was over a 2C TCR surface, as described in *A*. *D*, titration of binding of the indicated peptide/sH-2L^d complexes to a 2C TCR-coupled surface. The peptides were used at a large molar concentration (500 μM). The refractive index due to the bulk concentration of sH-2L^d was subtracted from the equilibrium values and curve fit as described in *Materials and Methods*. *A* and *B* are from the same experiment; *C* and *D* from two independent experiments. *E*, summary of binding data obtained from two independent experiments. The RU values obtained with H-2L^d/p2Ca and H-2L^d/pMCMV complexes were used as positive and negative controls, respectively. The data for the different variant peptides are presented as percent of binding observed with p2Ca peptide, as described in *Materials and Methods*. Negative values are occasionally observed when absolute binding units are compared with controls, due to incremental deterioration of the immobilized biosensor surface with numerous cycles of regeneration and binding.

peptide interactions, we tested whether blocking the contribution of CD8-class I interaction could differentiate between the two classes of peptides. We therefore analyzed the efficiency of target sensitization by various peptides in the presence of saturating concentrations of anti-CD8 mAb. For these studies, all peptides were used at a dose of 5 μM.

The results of this analysis demonstrate that target sensitization induced by variant peptides falls into three groups depending on susceptibility to inhibition by the anti-CD8 mAb, 53–6.7 (Table II). Group I contains peptides (including P2CA, A1, V1, I1, and Y4) that showed strong functional activities and were bound by sTCR. Lysis induced by those peptides was largely unaffected by the presence of anti-CD8 mAb, with the degree of inhibition generally falling below 20%. Group II includes four variant peptides with somewhat heterogeneous properties. Target sensitization by those peptides was inhibited by approximately 25 to 50% by anti-CD8 mAb. Three of those peptides (A2, L4, and E7) exhibited strong functional activities, while the fourth (N7) was intermediate. Moreover, while all four peptides formed complexes with sH-2L^d effectively, complexes with L4 or N7 failed to bind sTCR to a detectable level; however, complexes formed by the other two peptides (A2 and E7) were bound by sTCR (see below for discussion). Group III includes peptides showing either intermediate or weak functional activities in one or both assays and were not detectably bound by sTCR (e.g., A3, A7, and A8). The activity of those peptides was almost completely inhibited by anti-CD8 mAb (blocking >70%). The anti-CD8 mAb-mediated blocking is dose dependent and qualitatively similar data have been obtained using a second anti-CD8 mAb, 2.43 (data not shown). Taken together, these findings clearly show a distinction between the properties of the various peptides that most closely resembles the results of the direct sTCR binding studies. Furthermore, they provide direct evidence for a link between susceptibility to inhibition by anti-CD8 mAb and intrinsic TCR affinity.

Two exceptions to the above categorization of peptides were observed with the A2 and E7 variants. These peptides exhibited strong levels of functional activities and their complexes with sH-2L^d were bound, albeit to a lower degree, by 2C sTCR. Lysis of target cells induced by these peptides was, nevertheless, partially inhibited by anti-CD8

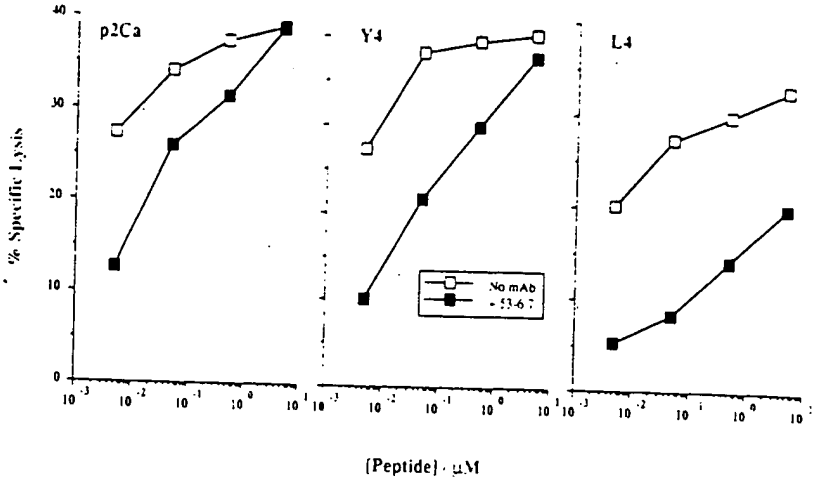

FIGURE 8. Effect of ligand density on susceptibility of 2C lysis to anti-CD8 mAb. Three peptides (p2Ca, Y4, and L4) were titrated at the indicated final concentrations in the presence or absence of 1/10 final dilution of 53–6.7 mAb supernatant. E:T ratio was 3:1. Background lysis (in the absence of peptide) was 3.2%.

mAb (Table II). Although the exact reason for this is unknown, it could be related to the slightly lower efficiency with which the two peptides bind sH-2L^d.

To further address the issue of the apparently contradictory findings, reported by others (21, 22), as to the CD8-dependence of 2C TCR recognition, we studied the effect of 53–6.7 mAb on target lysis induced by decreasing concentrations of peptides. Figure 8 illustrates representative results obtained using three different peptides, p2Ca and Y4 (both classified as strong sTCR binders and anti-CD8 mAb resistant: see above) and L4 which, despite exhibiting strong functional activity, is not detectably bound by TCR and is sensitive to anti-CD8 mAb blocking (Table II). At high concentrations of peptides (the highest dose used was 5 μM), lysis induced by p2Ca or Y4 peptide was not blocked by anti-CD8 mAb, while that induced by L4 peptide was inhibited by approximately 39% (Fig. 8). However, as the peptide dose decreased, lysis induced by all three peptides became progressively more susceptible to anti-CD8 mAb blocking. This reached a maximum at a dose of 5 nM, where the degree of inhibition observed with p2Ca, Y4, and L4 peptides was 54.0%, 63.4%, and 74.3%, respectively. In this regard, it is important to note that, for all three peptides, the dose required for half-maximal sensitization is about 3 nM, and plateau levels of lysis are reached at a dose of approximately 50 nM (Fig. 5*B*). These data demonstrate that, for a given CD8$^+$ TCR-MHC/peptide interaction, susceptibility to inhibition by anti-CD8 mAb is not only a function of the intrinsic affinity of the TCR to its ligand, but is also influenced by small alterations in the cell-surface density of the MHC/peptide complex.

Discussion

The T cell repertoire in mature animals is shaped by two selective events that take place within the thymus. During positive selection, thymocytes expressing receptors that recognize self-MHC molecules with low binding affinity are selected for further maturation and, ultimately, are exported to populate the periphery. In contrast, thymocytes bearing receptors that interact with self-MHC with an affinity above a certain threshold are subjected to negative selection, which ensures their deletion by apoptotic death in the thymus. Thus, for both of those processes, which lead to two completely divergent outcomes, the central determining factor is thought to be the strength of interaction between the TCR and MHC/peptide ligand. Recently, direct support for this hypothesis has been obtained from studies using in vitro fetal thymic organ cultures, which allow for direct monitoring of the fate of thymocytes of a given specificity in the presence of MHC/peptide ligands (37–39). These studies demonstrated that, in addition to the intrinsic affinity of the TCR to its ligand, thymic selection can also be influenced by ligand density. Thus, the current evidence supports a model of thymic maturation based on the avidity of TCR-MHC/peptide interaction, a term that encompasses the intrinsic affinity of TCR as well as ligand density (40).

In our laboratory, we have been studying the recognition properties of a TCR derived from an alloreactive CTL clone, 2C. This clone was derived from BALB.B (H-2^b haplotype) mice injected with P815 mastocytoma cells and BALB/c spleen cells (both of H-2^d haplotype) and is specific to H-2L^d plus the p2Ca peptide (13, 23). Interestingly, analysis of HPLC fractions obtained from spleens of mice of different haplotypes indicated that a peptide with similar properties to p2Ca is also present in H-2^b and H-2^k mice (13). In transgenic mice, the 2C TCR has been shown to be positively selected on H-2K^b and negatively selected on H-2L^d (18, 19). Whether the same, or a closely related, peptide is involved in both positive and negative selections of the 2C TCR is unknown. It is interesting to note that 2C cells are able to lyse H-2K^b-expressing targets but only in the presence of ~1000-fold higher concentrations of p2Ca (11) (our unpublished observations). Using a soluble form of this receptor, we have previously determined that the

interaction between 2C TCR and the H-2L^d p2Ca ligand is one of moderate affinity with a K_d of $\sim 10^{-7}$ M (12). In this study, we extended our analysis by examining 2C TCR recognition of monosubstituted variants of p2Ca as a first step in the definition of the role of peptides in positive vs negative selection of this receptor. More specifically, the present study has three aims: 1) to characterize the contribution of each residue of the p2Ca moiety to H-2L^d binding vs TCR recognition; 2) to examine whether, and how, mutations at putative TCR-contact sites affect the affinity of interaction between the TCR and its ligand; and 3) to study the dependency of 2C TCR recognition on CD8 function.

Analysis of the effects of various substitutions on peptide function indicates that positions 6 and 8 of the octamer p2Ca peptide are important for binding to H-2L^d. Based on sequences of peptides eluted from H-2L^d molecules, we have previously identified a binding motif for H-2L^d consisting of P at position 2 and a hydrophobic amino acid (L, M, or F) at the C terminus (7). The p2Ca peptide does not strictly adhere to this motif, and we have suggested that the similarity of S and P residues at position 2 and the retention of P within the peptide may contribute to the ability of this, and similar peptides, to bind H-2L^d (7). The finding that A2 peptide was only minimally affected in its binding to sH-2L^d indicates that S to A substitution at this position is well tolerated, perhaps because of the preservation of the adjacent P residue. In contrast, the demonstration that the A8 peptide is 20 to 60 times less effective in binding to sH-2L^d confirms the importance of the C-terminal hydrophobic residue to H-2L^d binding. In competition experiments, the A8 peptide binds sH-2L^d 100- to 500-fold less well than p2Ca (data not shown). These results are in essential agreement with recent findings from Sykulev et al. (11). Moreover, the significantly lower binding exhibited by the A6 peptide indicates that the F residue at position 6 plays an important role in peptide binding, and may function as an anchor. The alanine scan analysis thus indicates that, for the p2Ca octamer peptide, positions 6 and 8 serve as primary anchors.

Examination of the properties of position 1 variant peptides indicates that the majority of substitutions at this residue are well tolerated, both at the level of binding to sH-2L^d and in functional activity. The one exception to this was the observation that the D1 peptide exhibited a small, but significant, reduction in activity in both the lysis and IL-3 assays. The decreased activity of D1 is likely to be due to its marginally reduced binding to sH-2L^d. Nevertheless, all position 1-substituted peptide/MHC complexes were bound, albeit to varying degrees, by sTCR indicating that substitutions at this site have only a minimal influence on peptide properties.

Analysis of variant peptides with substitutions at residues 3, 4, or 5 of p2Ca demonstrates that these positions are critical for TCR recognition. Compared with p2Ca, peptides substituted at any of these positions exhibited equivalent or, in some cases, superior binding to sH-2L^d. However, as observed with A3, A4, C4, and A5 variant peptides, these substitutions severely restricted TCR recognition. This was demonstrated both at the level of biologic function (IL-3 and/or lysis assays) and, more directly, by measurement of TCR-H-2L^d peptide binding by SPR. A recent report by Sykulev et al. (11) described similar findings for the A3 and A5 variant peptides. Furthermore, our data indicate that with some position 4 or 5 variant peptides (e.g., A4, C4, A5), a profound loss in functional activity is observed in the face of enhanced binding to H-2L^d. This suggests that, in these cases, reduced T cell recognition may be due not only to loss of putative TCR contact sites, but also to peptide-induced conformational changes in the H-2L^d-peptide complex. Assessment of position 7-substituted peptides also indicates that the major effect of this residue is on TCR recognition; however, some nonconservative substitutions at this position, as evidenced by K7 and T7 peptides, also have a significant influence on binding to H-2L^d. Thus, the negatively charged side chain (D) at position 7 may contribute to the stability of peptide binding to H-2L^d as well as to T cell recognition.

It has been generally assumed that differences in the affinity of TCR-MHC/peptide interaction would be manifested in the strength of biologic functions induced by the peptides (11, 41). Our data lend direct support for this model inasmuch as peptides with weak functional activity formed complexes with sH-2L^d that were not bound by sTCR. Similarly, variant peptides, such as Y4, I1, and V1, which exhibit wild-type levels of activity in functional assays formed complexes that were bound by sTCR to a degree comparable with that observed with p2Ca. However, as evidenced by the properties of several peptides, most notably L4, the above model does not appear to be a generalizable one. Thus, compared with p2Ca, the L4 peptide exhibited stronger binding to sH-2L^d and equivalent, or better, functional activity in both lysis and IL-3 assays. In the TCR-binding assay, however, L4/sH-2L^d complexes were not detectably bound by sTCR. These findings demonstrate a clear discordance between the intrinsic affinity of TCR to a particular H-2L^d/peptide complex and the ability of the complex to activate T cells.

The most general explanation for this discordance is that our purified MHC and TCR molecules do not completely mimic the interaction of the natural cell-surface molecules, and that they may lack structures critical for the cell-surface events. Nevertheless, the results with L4 peptide are most readily explained by invoking a role for other direct, or indirect, interactions of the MHC/peptide complex with cell surface components of the T cell. The most obvious candidate is the CD8 co-receptor. Thus, the observed wild-type level of activation in functional assays, in the face of an intrinsically lower TCR affinity, could be due to CD8-mediated enhancement of TCR-MHC/peptide interaction. Indeed, analysis of the susceptibility of 2C

CTL recognition of different MHC.peptide ligands to blocking by anti-CD8 mAb confirms this prediction. The data with L4 peptide, therefore, illustrate an important point: namely that, for a given interaction, the critical parameter determining whether or not a response is induced appears to be the attainment of a minimum threshold of total avidity irrespective of whether the intrinsic TCR affinity is 10^{-7} or 10^{-5} M. In this case, the contribution of CD8 co-receptor appears to be more than sufficient to offset the differences in affinity.

Target cell sensitization induced by the "high affinity" group of peptides became susceptible to anti-CD8 mAb inhibition when lower doses of peptides were used. Thus, at a dose of 50 nM, which normally resulted in near plateau levels of lysis, the response with p2Ca and Y4 peptides was inhibited by ~24% and 44%, respectively. This clearly demonstrates that CD8-dependence is influenced not only by the intrinsic affinity of TCR but also by ligand density. The influence of ligand density on CD8-dependence of effector CTL function has been previously reported (42, 43). CD8 involvement becomes crucial in situations in which the total avidity of TCR-MHC/peptide interactions is less than optimal. As our data directly demonstrate, such situations could arise as a result of either low TCR affinity/high ligand density, or high TCR affinity/low ligand density, interactions.

Although the compensation for lack of binding affinity by CD8 effects may in part explain the behavior of the L4 peptide, we must still explain how other peptides whose complexes fail to bind the TCR can stimulate the T cell. One model for the variable functional effectiveness of different peptides whose complexes are not bound by the TCR is that different peptides have distinct abilities in the generation of high affinity interactions between the MHC and CD8. Although no current evidence indicates any peptide-dependent change in CD8/MHC affinity, this has not yet been directly demonstrated. Indirect support for this model comes from recent findings showing that mutations in the $\alpha 2$ domain of MHC class I molecules influence binding to CD8 (P. Kavathas, personal communication). An alternative model would not invoke CD8 at all, but merely suggest that two sequential steps are needed for T cell activation: first, the binding of the MHC/peptide complex to the TCR and, second, some additional conformational event. Some MHC/peptide complexes may do both well, while others fail to do either. Those that do the first poorly (i.e., do not bind well) but do the second effectively could be nonbinding, active complex formers. Those that lack whatever structural requirements are needed for the critical second step might bind well but not activate. In support of this model, a recent study demonstrated that the ability of various anti-TCR Abs to act as agonists vs antagonists appeared to be dependent on their capacity to induce the recruitment of CD4 and CD45 molecules into the TCR signaling complex (44).

In conclusion, the results described in this report demonstrate four points. 1) Substitutions at critical TCR contact sites of an antigenic peptide yield variants that are recognizable with lower TCR affinity. 2) Some of these "low affinity" variant peptides can still induce strong functional responses, comparable with p2Ca peptide; thus, the strength of a functional in vitro response does not always correlate with intrinsic affinity of the TCR to MHC/peptide complex. 3) For a given CTL target interaction, dependence on the CD8 co-receptor is a function of at least two parameters: intrinsic TCR affinity and ligand density. 4) Our findings suggest that the MHC/peptide complex forms a structure that signals the TCR not only by binding, but also by additional parallel or distal events.

Acknowledgments

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